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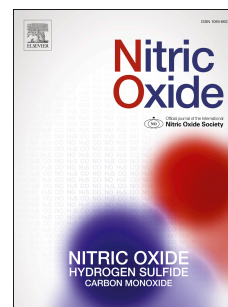
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# Accepted Manuscript

Chronic high-dose beetroot juice supplementation improves time trial performance of well-trained cyclists in normoxia and hypoxia

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1     **Title**

2     **Chronic high-dose beetroot juice supplementation improves time trial**  
3     **performance of well-trained cyclists in normoxia and hypoxia**

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18    Declarations of interest: EW is a co-applicant on patents related to the therapeutic  
19    use of nitrate and nitrite. Other authors, none.

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## Abstract

Dietary nitrate ( $\text{NO}_3^-$ ) supplementation via beetroot juice (BR) is known to improve endurance performance in untrained and moderately trained individuals. However, conflicting results exist in well-trained individuals. Evidence suggests that the effects of  $\text{NO}_3^-$  are augmented during conditions of reduced oxygen availability (e.g., hypoxia), thereby increasing the probability of performance improvements for well-trained athletes in hypoxia vs. normoxia. This randomized, double-blinded, counterbalanced-crossover study examined the effects of 7 days of BR supplementation with 12.4 mmol  $\text{NO}_3^-$  per day on 10-km cycling time trial (TT) performance in 12 well-trained cyclists in normoxia (N) and normobaric hypoxia (H). Linear mixed models for repeated measures revealed increases in plasma  $\text{NO}_3^-$  and  $\text{NO}_2^-$  after supplementation with BR (both  $p < 0.001$ ). Further, TT performance increased with BR supplementation ( $\sim 1.6\%$ ,  $p < 0.05$ ), with no difference between normoxia and hypoxia ( $p = 0.92$ ). For respiratory variables there were significant effects of supplementation on  $\text{VO}_2$  ( $p < 0.05$ ) and VE ( $p < 0.05$ ) such that average  $\text{VO}_2$  and VE during the TT increased with BR, with no difference between normoxia and hypoxia ( $p \geq 0.86$ ). We found no effect of supplementation on heart rate, oxygen saturation or muscle oxygenation during the TT. Our results provide new evidence that chronic high-dose  $\text{NO}_3^-$  supplementation improves cycling performance of well-trained cyclists in both normoxia and hypoxia.

Keywords:

Nitrate,

Nitrite,

Endurance exercise,

Cycling performance,

Hypoxia,

## 57 1.1 Introduction

58 There is general consensus regarding the physiological factors that limit  
 59 endurance performance [1,2]. These factors include maximal oxygen consumption  
 60 ( $\text{VO}_{2\text{max}}$ ), the fractional utilization of  $\text{VO}_{2\text{max}}$ , and exercise efficiency. Even  
 61 minor improvements in these factors can enhance performance of endurance  
 62 athletes. One strategy proposed to improve performance is inorganic nitrate ( $\text{NO}_3^-$   
 63 ) supplementation, most often in the form of concentrated beetroot juice (BR) [3].  
 64 When ingested, nitrate is reduced to nitrite and nitric oxide (NO). This pathway  
 65 differs from the classical pathway for NO generation which involves specific  
 66 enzymes, NO-synthases (NOS) that use L-arginine and molecular oxygen to  
 67 generate NO. Nitric oxide has been demonstrated to alter several physiological  
 68 processes such as blood flow, mitochondrial function and contractile properties  
 69 [3-8]. Recently, several studies have provided evidence that dietary intake of  $\text{NO}_3^-$   
 70 can improve exercise efficiency (reduction in  $\text{VO}_2$  at same work rate) [9-12] and  
 71 endurance performance [9,10,13-17]. Notably, the majority of studies reporting  
 72 beneficial effects of  $\text{NO}_3^-$  has been conducted in untrained and moderately trained  
 73 individuals ( $\text{VO}_{2\text{max}} < 60 \text{ ml/min/kg}$ ) [10,15,16,18], whereas studies in highly  
 74 trained individuals ( $\text{VO}_{2\text{max}} > 60 \text{ ml/min/kg}$ ) have shown minor [16,19-21] or no  
 75 improvements [22-27], indicating that  $\text{NO}_3^-$  may be less effective in this  
 76 population [28,29]. In addition to this, recent studies in hypoxia have also  
 77 provided evidence that  $\text{NO}_3^-$  improves exercise efficiency [17,21,30,31], muscle  
 78 oxygenation [31] and elevates oxygen saturation ( $\text{SpO}_2$ ) [21,30,31]. The lower  $\text{O}_2$   
 79 availability in hypoxia impairs the L-Arginine-NOS pathway, and potentiates the  
 80 nitrate-nitrite-NO pathway, suggesting that BR may be more effective in hypoxia

81 than in normoxia [3,32-34]. Supporting the notion that BR is more effective in  
82 hypoxia, Kelly et al. [30] showed that, in healthy individuals, BR improved time  
83 to exhaustion during severe intensity exercise in hypoxia but not in normoxia. In  
84 addition, BR has been shown to attenuate the decrease in muscle oxygenation and  
85 muscle metabolic perturbation in hypoxia in untrained and moderately trained  
86 subjects [31,35]. Hence, highly trained athletes may also experience greater  
87 performance improvements with BR in hypoxia compared with normoxia.  
88 Recently, few studies have examined this idea with conflicting results. In well-  
89 trained athletes  $\text{NO}_3^-$  supplementation had no effect on 10-km or 15-km cycling  
90 performance, 10-km running performance or roller-skiing treadmill performance  
91 in hypoxia [36-39]. Contrary to this, two studies have reported positive effects of  
92 BR in hypoxia on 16.1-km cycling performance and 1500m running performance  
93 in trained athletes [17,21]. The discrepancy could be due to different  
94 supplementation strategies for  $\text{NO}_3^-$ . Specifically, the effects of  $\text{NO}_3^-$   
95 supplementation seems to be potentiated with BR as source of  $\text{NO}_3^-$  [40,41], with  
96 chronic loading over several days [42,43], and by using a dose of >8mmol per day  
97 [13,20,44]. Optimizing the supplementation strategy of  $\text{NO}_3^-$  may be even more  
98 important in trained athletes, as this population already exhibit adaptations elicited  
99 by endurance training and diet, including higher  $\text{NO}_3^-$  plasma levels [45,46], NO  
100 release [47], NOS activity[48] and a higher percentage of type I fibers [8,49], that  
101 altogether may attenuate the response to  $\text{NO}_3^-$  supplementation.  
102 The purpose of the present study was to examine the effects of several days  
103 supplementation with a high-dose BR on cycling time trial performance in well-  
104 trained cyclists, with continuous measurements of  $\text{SpO}_2$ , muscle oxygenation and

oxygen uptake in normoxia and normobaric hypoxia. We hypothesized that BR would improve TT cycling performance in hypoxia but not in normoxia.

## 2.1 Material and Methods

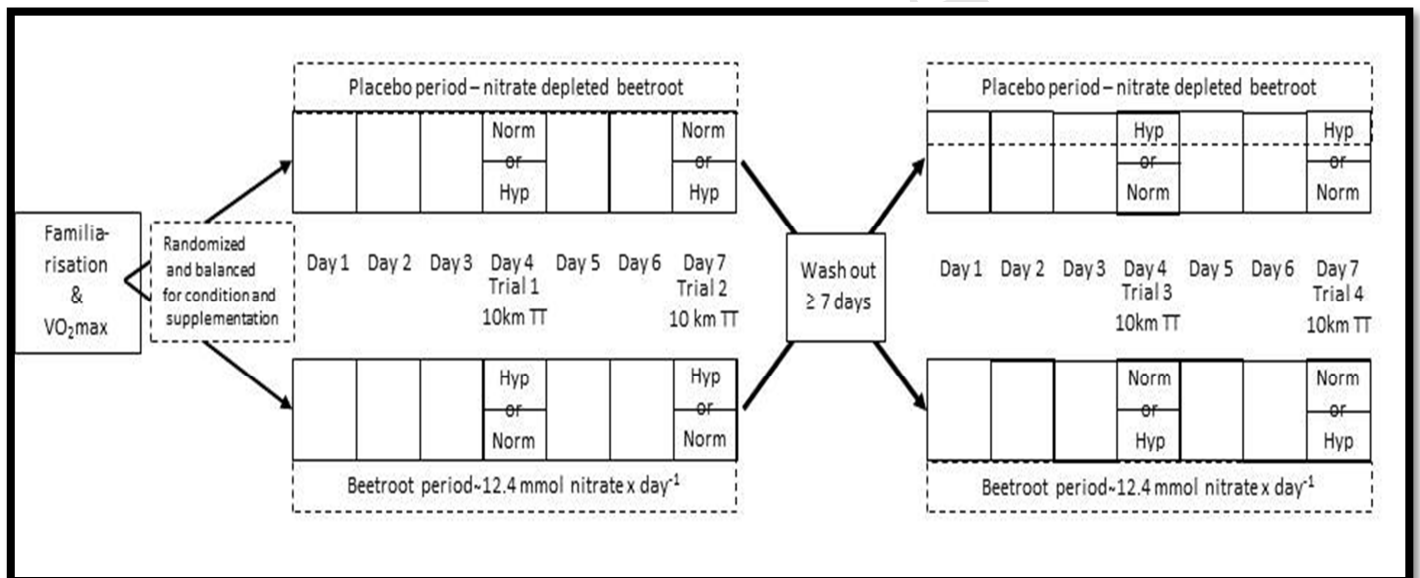
### 2.1.1 Participants

Twelve healthy male cyclists at the age of  $29.1 \pm 7.7$  yrs (range 22 to 44 yrs) were enrolled in the study. Participants had a  $\text{VO}_{2\text{max}}$  of  $5.09 \pm 0.47 \text{ L}\cdot\text{min}^{-1}$  corresponding to  $66.4 \pm 5.3 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$  and a wattmax of  $430 \pm 35 \text{ watt}$  corresponding to  $5.6 \pm 0.3 \text{ watt}\cdot\text{kg}^{-1}$  (mean  $\pm$  SD). Participants were best classified as well-trained in performance level 4 as defined by Jeukendrup et al. [50] and De Pauw et al. [51], respectively. The protocol and test procedures used in the current study were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Northern Jutland (N-20150049). All participants signed informed consent prior to enrollment.

### 2.1.2 Study design

Participants reported to the laboratory on five separate occasions. Experimental trials followed a randomized counterbalanced-crossover design and were double-blinded for supplementation and single-blinded for inspiratory conditions. The first visit consisted of a maximal exercise performance test to ensure participants were familiar with testing procedures and to ensure participants met the inclusion criteria (i.e.,  $\text{VO}_{2\text{max}} > 60 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  or wattmax  $\geq 5 \text{ w/kg}$ ). Visits 2-5 involved four experimental trials (Fig 1). Each trial consisted of a 10-km time trial performed in conditions of normoxia or hypoxia, with supplementation of BR or nitrate-depleted BR as placebo (PLA). Specifically, supplementations were ingested in periods of seven days, separated by a wash out period of at least seven

129 days. During each supplementation period, 10-km time trials were performed on  
 130 day four and day seven, in different conditions. The order of condition was  
 131 maintained for each individual for the first and second supplementation period  
 132 such that visits 1 and 3 (and visit 2 and 4) were performed in the same condition.  
 133 The design was counterbalanced for condition and supplementation such that half  
 134 of the participants started with normoxia and half of the participants started with  
 135 BR. All exercise trials were performed on the Cyclus2 ergometer (RBM Cyclus 2,  
 136 Germany) using the participants' own bike.



137 **Figure 1: Experimental design**

### 138 2.1.3 Maximal exercise performance

139 Participants completed a 10-minute warm up at 100 watts and hereafter an  
 140 incremental exercise test to exhaustion to determine gas exchange threshold  
 141 (GET[30]),  $VO_{2max}$  and wattmax (Fig 1). The incremental exercise test  
 142 commenced at 100 watts and increased by 30 watts each minute until voluntary  
 143 exhaustion. Following a 10-minute rest, participants completed a familiarization  
 144 trial for the 10-km TT. While a  $VO_{2max}$  validation bout is recommended [52], this



was not performed in this present study as these well-trained cyclists routinely achieve maximal effort during exercise. Respiratory breath-by-breath data were measured throughout the test using a metabolic cart (Jaeger, Vyntus CPX, Carefusion). The metabolic cart was calibrated before each test according to the manufacturer's recommendations. Maximal oxygen uptake ( $\text{VO}_{2\text{max}}$ ) was determined as the highest 30-second average, Wattmax as peak power output from the last minute of the test ( $((\text{watt}) + \text{time in last stage (s)}/60 \times 30 (\text{W}))$ ) and heart rate (HR) as the peak value attained during the test. GET was determined from a number of measurements, including 1) the first disproportionate increase in  $\text{VCO}_2$  from visual inspection of plotting  $\text{VCO}_2$  and  $\text{VO}_2$  and 2) an increase in expired ventilation ( $\text{V}_\text{E}/\text{VO}_2$ ) with no increase in  $\text{V}_\text{E}/\text{VCO}_2$  [30]. HR was recorded continuously using a heart rate sensor (Polar Electro, Oy, Finland).

#### 2.1.4 Experimental trials

Participants ingested BR or PLA for seven consecutive days (Fig 1). Specifically, participants consumed 140ml of concentrated BR (~12.4 mmol nitrate) or 140ml of nitrate-depleted BR (PLA; ~0 mmol nitrate) (Beet It Sport, James White Drinks Ltd., Ipswich, UK) per day; one dose (70 ml) in the morning and one dose (70 ml) in the evening. On the days of the experimental trials (i.e., days four and seven), participants were instructed to consume the total dose (i.e., 140 ml) 2-h prior to arriving at the laboratory (approx. 2.75-h. before commencing the time trial). During the 24-h preceding the first experimental trial, each participant recorded their diet and was told to replicate this diet for the remaining three trials. Participants were also instructed to avoid the intake of specific nitrate-rich foods.

169 The use of antibacterial mouthwash products was not permitted and caffeine  
170 intake was prohibited for 12-h preceding each test. For each individual, all  
171 experimental trials were performed at the same time of day.

172 Upon arrival at the laboratory, participants rested for 5-minutes before a resting  
173 blood sample was drawn into two 4 ml lithium heparin vacutainers  
174 (Becton Dickinson, Plymouth, UK). Blood samples were immediately centrifuged  
175 for 10 min at 4°C, 3000g after which plasma was extracted and stored at -80 °C  
176 for later determination of plasma nitrate and nitrite according to the method  
177 described by Hezel et al. [53]. A near infrared spectroscopy (NIRS) probe  
178 (Oxymon MK III, Artinis Medical Systems, Netherlands) was placed on the belly  
179 of the Vastus Lateralis of the right leg in order to measure changes in muscle  
180 oxygenation. Probe position was marked with a permanent pen to ensure identical  
181 probe placement for subsequent trials, and the NIRS probe was placed with  
182 double-sided adhesive tape. Further, elastic bandages were used to ensure a fixed  
183 placement of the probe. An earlobe pulse oximeter (Nonin XPod 8000Q2, Nonin  
184 Medical, Inc, Plymouth, MN) was used to measure SpO<sub>2</sub> throughout the tests.

185 Participants then rested 5-minutes on the bike while breathing the gas mixture  
186 corresponding to the condition for that specific trial. Throughout each trial,  
187 participants breathed through a facemask (Hans Rudolph, V-982185) connected to  
188 a low resistance y-valve (Hans Rudolph, two way Y-shape non-rebreathing valve,  
189 2730L), with the inspiration valve connected to a closed reservoir. The inspired  
190 gas was modified via the closed reservoir using a custom built setup consisting of  
191 a mechanical ventilator (SV-300, Maquet, Solna, Sweden) modified such that  
192 mixing of gas (pressurized room air and nitrogen) was controlled by manipulating

the inspired oxygen setting on the ventilator. The participants breathed through the same circuit for all experimental trials. The fraction of inspired oxygen was adjusted to  $15 \pm 0.1\%$  in hypoxia (~2500m of altitude) and  $20.9 \pm 0.1\%$  in normoxia (sea level). Warm-up consisted of three six-minute exercise bouts at the power output corresponding to 70% of GET measured in normoxia. A six-minute rest separated each bout. After the third bout, participants rested for 10 minutes without the facemask. Prior to the TT, participants sat on the bike for five minutes while breathing the gas mixture corresponding to the conditions for that specific trial. Then participants completed a 10-km TT with the instruction of finishing with the highest average power output and as fast as possible. Participants were blinded to all information except cadence and remaining distance of the TT, and were verbally encouraged at each km completed.  $\text{VO}_2$  and HR were measured continuously during the TT. For all physiological variables, average values from the 10km-TT were calculated and used for further analyses. Further, peak values for  $\text{VO}_2$ , RER (both highest 30-s average) and HR (highest 1-s value) during the TT were calculated and used for further analyses. The ratio of average power to average oxygen uptake ( $\text{PO}/\text{VO}_2$ ) during the time trial was used as an index of exercise efficiency [15]. NIRS variables of oxygenated ( $\text{HbO}_2$ ), deoxygenated (HHb) and total (THb) hemoglobin were recorded continuously at 2 Hz and expressed as relative changes ( $\Delta$ ) from the baseline value measured during the final 90-seconds pre-exercise rest period.

#### 2.1.5 Statistical analysis

Differences in performance and physiological parameters were analyzed using linear mixed models for repeated measures. This method of data analysis was

used as it has the advantage of preventing listwise deletion due to missing data (md). For clarification, md for each variable has been noted in table 1. As the dependent variable, the variable of interest was entered (watt,  $\text{VO}_2$ , VE,  $\text{VCO}_2$ ,  $\text{SpO}_2$ , etc.) into the model. To investigate the effects of supplementation (BR vs. PLA), condition (hypoxia vs. normoxia) and supplementation-by-condition, these were entered as fixed effects. Subject id was included in the model as a random effect to control for the within-subject nature of the 4 trials. Further, paired t-tests were used to compare differences between the  $\text{VO}_{2\text{peak}}$  obtained during the normoxic time trials and the  $\text{VO}_{2\text{max}}$  from the ramp incremental test. Within group effect sizes were calculated as the difference in means (BR vs. PLA) divided by the pooled SD of the change score, using the following definitions: trivial effect  $d < 0.2$ , small effect  $> 0.2$ , moderate effect  $> 0.5$ , large effect  $> 0.8$  [54].

Associations between changes in TT performance and changes in  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{VO}_2$ , and  $\text{SpO}_2$  from PLA to BR were assessed using Pearson correlation coefficient.

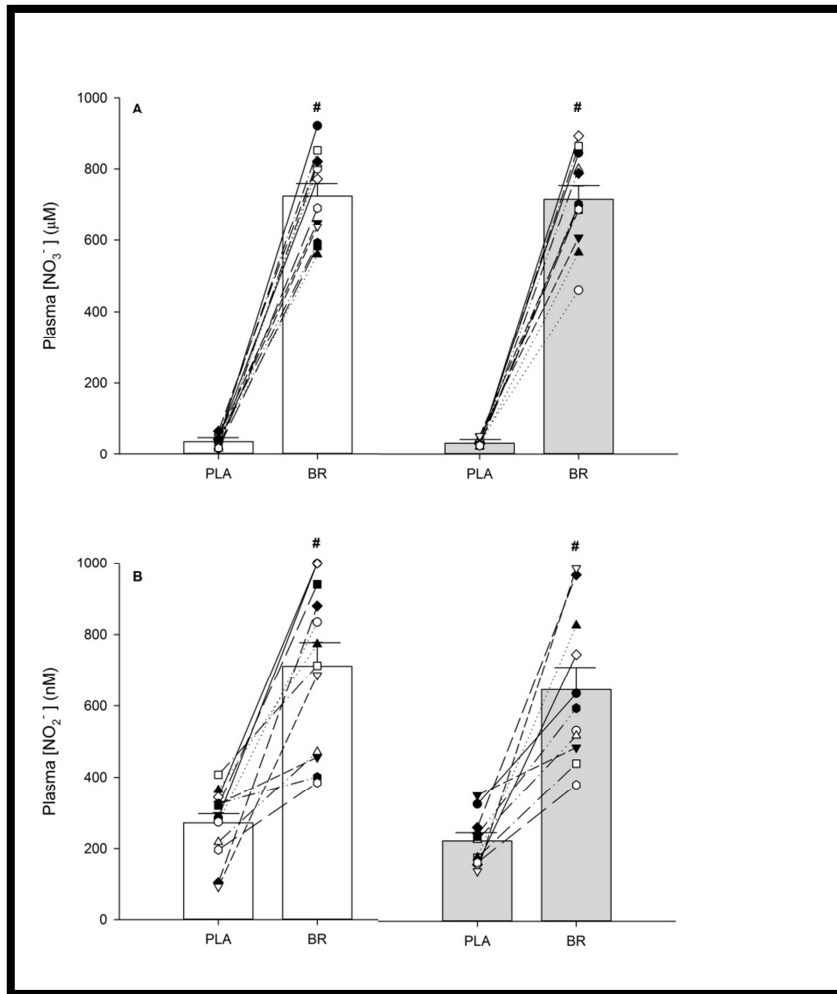
All data are presented as means  $\pm$  SE, unless stated otherwise, with statistical significance being accepted when  $P \leq 0.05$ . All statistical tests were performed using SPSS 25 (IBM Corp., Armonk, USA) or STATA (Texas, USA) version SE 12.1.

### 3.1 Results

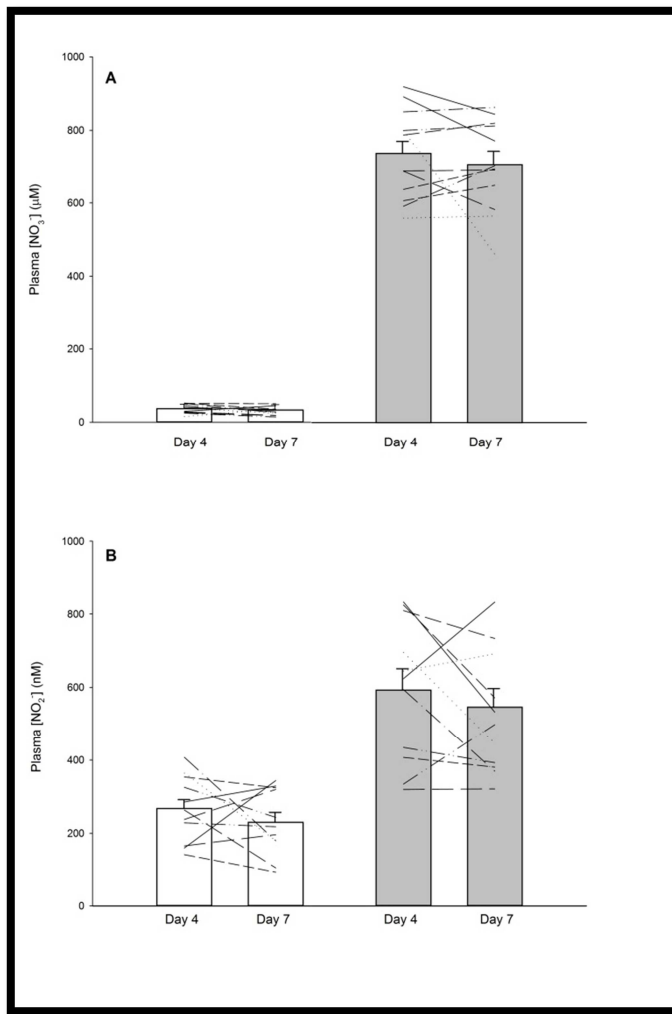
#### 3.1.1 Plasma nitrate and nitrite

There were significant main effects of supplementation on  $\text{NO}_3^-$  and  $\text{NO}_2^-$  (both  $p < 0.001$ ) such that BR elevated  $\text{NO}_3^-$  and  $\text{NO}_2^-$  (Fig 2). There were no effects of

condition ( $\text{NO}_3^-$   $p=0.858$ ;  $\text{NO}_2^-$   $p=0.542$ ) or supplementation-by-condition  
 interaction ( $\text{NO}_3^-$   $p<0.907$ ;  $\text{NO}_2^-$   $p=0.687$ ).  
 Further, there were no differences in levels of  $\text{NO}_3^-$  ( $p=0.234$ ) or  $\text{NO}_2^-$  ( $p=0.231$ )  
 between 4 and 7 days of supplementation (Fig 3).



**Figure 2: Individual and mean plasma levels of  $\text{NO}_3^-$  (A) and  $\text{NO}_2^-$  (B) (mean $\pm$ SE) prior to time trial tests in normoxia (open bars) and hypoxia (filled bars), after supplementation with beetroot juice (BR) or placebo (PLA). (#,  $p < 0.001$ , PLA vs. BR, N=11 in hypoxic conditions).**

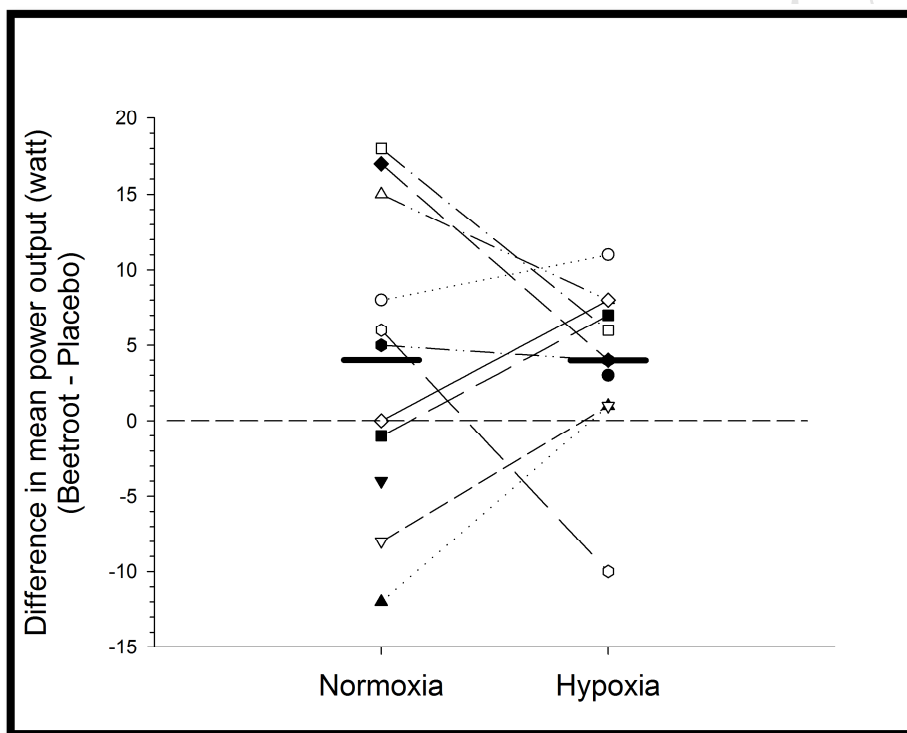


**Figure 3: Individual and mean plasma levels of NO<sub>3</sub><sup>-</sup> (A) and NO<sub>2</sub><sup>-</sup> (B) (mean±SE) prior to time trial tests at day 4 and day 7 after supplementation with beetroot juice (filled bars) or placebo (open bars). (#,  $p < 0.001$ , PLA vs. BR, N=11 in hypoxic conditions).**

### 3.1.2 Time trial performance

All participants completed all four TT's. However, two tests were discarded due to measurement error (n=1 in N-BR and n=1 in H-PLA). Time trial performance data are presented in Table 1. There was a main effect of condition ( $p < 0.001$ ) on time trial performance such that hypoxia lowered power output by ~15% and ~6%, respectively. Further, there was a main effect of supplementation on time trial power output ( $p = 0.019$ ) and completion time ( $p = 0.024$ ) showing an overall 1.6%

263 increase in power output and 0.6% reduction in completion time with BR (Fig 4),  
 264 with no condition-by-supplementation interaction (both  $p=0.923$ ). Notably, 10 out  
 265 of 11 participants increased power output in H-BR compared to H-PLA, whereas  
 266 6 out of 11 increased power output in N-BR compared to N-PLA (Fig 4). Effect  
 267 size calculations for within group differences between BR and PLA show  
 268 moderate (0.703) and small (0.398) effects for hypoxia and normoxia,  
 269 respectively.



270  
 271 **Figure 4. Individual and mean differences in power output (watt) during 10 km TT**  
 272 **performance between placebo and beetroot supplementations in normoxic and hypoxic**  
 273 **conditions. Bold horizontal lines indicate mean values for each condition. Single dotted line**  
 274 **indicates no difference between beetroot and placebo supplementation**

		Md	N-PLA	N-BR	H-PLA	H-BR	Linear mixed model effects		
							Supplement	Condition	Interaction
<u>Time Trial</u>									
<b>Performance variable</b>									
Power output, Watt	2	311.3 ± 13.2	315.8 ± 13.2	264.4 ± 13.2	269.3 ± 13.2	p=0.019	p<0.001	p=0.923	
Completion time, sec	2	890.1 ± 16	884.5 ± 16	945.6 ± 16	939.5 ± 16	p=0.024	p=0.001	p=0.923	
<b>Average values</b>									
PO/VO <sub>2</sub> , W/L <sup>-1</sup> ·min <sup>-1</sup>	10	71.1 ± 1.8	70.8 ± 1.8	68.0 ± 1.8	68.0 ± 1.8	p=0.777	p=0.001	p=0.757	
VO <sub>2</sub> , ml· min <sup>-1</sup>	10	4364 ± 140	4443 ± 139	3855 ± 142	3948 ± 142	p=0.030	p<0.001	p=0.862	
% VO <sub>2max</sub>	10	85.9 ± 1.6	87.4 ± 1.6	75.8 ± 1.7	77.7 ± 1.7	p=0.038	p<0.001	P=0.798	
VCO <sub>2</sub> , ml· min <sup>-1</sup>	10	4300 ± 151	4498 ± 150	4012 ± 153	4067 ± 153	p=0.005	p<0.001	P=0.120	
VE, L· min <sup>-1</sup>	10	129.9 ± 7.0	135.8 ± 7.0	136.4 ± 7.1	142.4 ± 7.1	p=0.019	p=0.010	P=0.998	
RER	10	0.99 ± 0.01	1.01 ± 0.01	1.04 ± 0.01	1.03 ± 0.01	p=0.462	p=0.003	P=0.082	
HR· min <sup>-1</sup> ,	3	168.5 ± 3.1	171.2 ± 3.1	169.4 ± 3.1	169.5 ± 3.1	p=0.118	p=0.486	P=0.072	
SpO <sub>2</sub> , %	9	97.1 ± 0.9	97.1 ± 0.9	84.5 ± 0.9	84.3 ± 0.9	p=0.787	p=0.000	P=0.779	
<b>Peak values</b>									
VO <sub>2peak</sub> , ml· min <sup>-1</sup>	10	4925 ± 151	4895 ± 150	4225 ± 152	4304 ± 152	p=0.443	p<0.001	p=0.111	
HR <sub>peak</sub> , min <sup>-1</sup>	3	183.9 ± 2.9	185.5 ± 2.9	181.1 ± 2.9	181.5 ± 2.9	p=0.153	p<0.001	p=0.308	
RER <sub>peak</sub>	10	1.07 ± 0.02	1.1 ± 0.02	1.14 ± 0.02	1.14 ± 0.02	p=0.334	p=0.003	p=0.246	
<b>NIRS</b>									
ΔHbO <sub>2</sub> , AU	3	-28.5 ± 2.6	-27.6 ± 2.6	-30.7 ± 2.6	-29.4 ± 2.6	p=0.543	p=0.061	p=0.849	
ΔHHb, AU	3	24.5 ± 2.6	23.9 ± 2.6	26.3 ± 2.6	26.6 ± 2.6	p=0.885	p=0.042	p=0.633	
ΔTHb, AU	3	-4.3 ± 2.0	-3.4 ± 2.0	-3.9 ± 2.0	-2.7 ± 1.9	p=0.527	p=0.766	p=0.934	
ΔHHb/VO <sub>2</sub> , AU· L·min <sup>-1</sup>	12	5.68 ± 0.73	5.75 ± 0.71	7.01 ± 0.78	6.78 ± 0.74	p=0.851	p=0.017	p=0.728	

279 **Table 1- Average and peak performance, ventilatory and cardiopulmonary data during the**  
280 **TT. md denotes the number of missing data points from each variable (complete number of**  
281 **data points = 48).**

282 *3.1.3 TT physiological data*

283 Physiological data obtained during the TT are presented in Table 1. There were  
284 significant effects of condition on SpO<sub>2</sub> (p<0.001), VE (p=0.010), RER

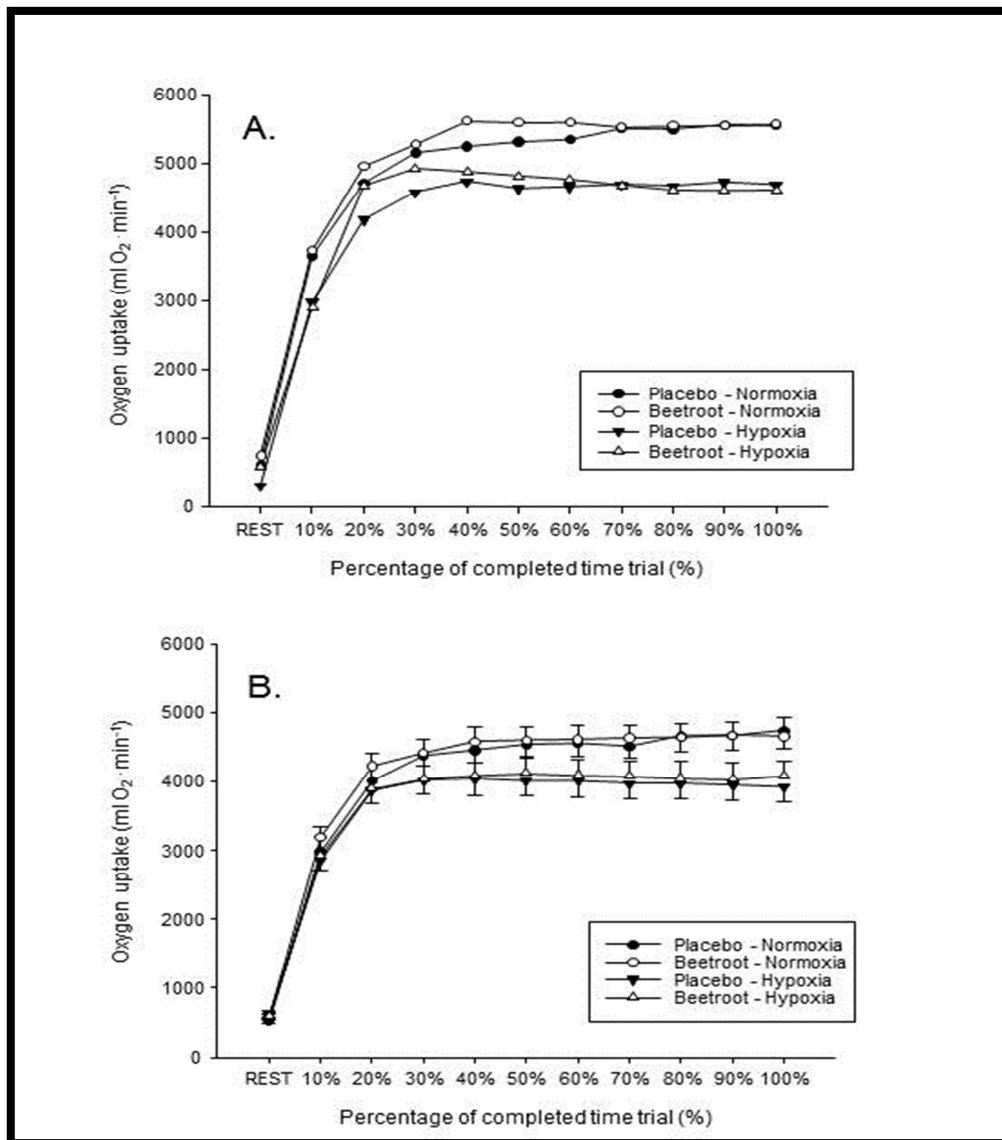


285 (p=0.003),  $\text{VCO}_2$  (p=0.001),  $\text{VO}_2$  (p<0.001),  $\text{PO}/\text{VO}_2$  (p=0.001) and %  $\text{VO}_{2\text{max}}$   
286 (p<0.001) such that hypoxia decreased  $\text{SpO}_2$ ,  $\text{VCO}_2$ ,  $\text{VO}_2$ ,  $\text{PO}/\text{VO}_2$ ,  $\text{VO}_{2\text{peak}}$ ,  
287  $\text{HR}_{\text{peak}}$  and %  $\text{VO}_{2\text{max}}$  while  $\text{VE}$ ,  $\text{RER}$  and  $\text{RER}_{\text{peak}}$  increased. There were  
288 significant effects of supplementation on  $\text{VO}_2$  (p=0.030) (Fig 5),  $\text{VE}$  (p=0.019),  
289  $\text{VCO}_2$  (p=0.005) and %  $\text{VO}_{2\text{max}}$  (p=0.038) such that  $\text{VO}_2$ ,  $\text{VE}$ ,  $\text{VCO}_2$  and %  $\text{VO}_{2\text{max}}$   
290 increased with BR. The  $\text{VO}_{2\text{peak}}$  attained during the time trials in normoxia were  
291 significantly lower than the  $\text{VO}_{2\text{max}}$  measured from the incremental test (N-PLA  
292 ~3.3%, p=0.03; N-BR ~3.7%, p=0.02).

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296

297 **Figure 5- Oxygen uptake profiles from an exemplar subject (A) and mean data (B) from all**  
 298 **conditions.**

299

### 300 3.1.4 Near infrared spectroscopy measures of muscle oxygenation

301 Data reflecting changes in muscle oxygenation during the TT are presented in

302 Table 1. There was a main effect of condition on  $\Delta\text{HHb}$  ( $p=0.042$ ) and

303  $\Delta\text{HHb}/\text{VO}_2$  ( $p=0.017$ ) such that the increase in  $\Delta\text{HHb}$  and  $\Delta\text{HHb}/\text{VO}_2$  during the

304 TT was greater in hypoxia (Table 1). We also found a near-significant main effect

of condition on  $\Delta\text{HbO}_2$  ( $p=0.061$ ) indicating a greater reduction of  $\Delta\text{HbO}_2$  during TT in hypoxia.

### 3.1.5 Correlations

There were no significant correlations between changes in performance and changes in plasma  $\text{NO}_3^-$  or  $\text{NO}_2^-$  after BR supplementation in normoxia or hypoxia. Further, there were no significant correlations between changes in performance (BR vs. PLA) and changes in  $\text{VO}_2$  or  $\text{SpO}_2$  nor between changes in performance (BR vs. PLA) and  $\text{VO}_{2\text{max}}$ .

## 4.1 Discussion

This is the first study to examine the effects of chronic supplementation with high-dose  $\text{NO}_3^-$ , in the form of BR, on time trial performance in well-trained athletes in both hypoxia and normoxia. We show a significant main effect of BR on 10-km TT performance, indicating that well-trained cyclists improve power output and completion time with BR in both normoxia and hypoxia. Supplementation with BR also increased  $\text{VO}_2$  during the TT in hypoxia and normoxia, showing that the participants were able to utilize a higher fraction of  $\text{VO}_{2\text{max}}$  with BR.

### 4.1.1 Effects of BR supplementation on TT performance

We found a main effect of BR supplementation on TT performance with no condition-by-supplementation interaction, indicating that BR increased TT performance with no difference between hypoxia and normoxia. However, from a practical perspective, it is worth highlighting that 10 out of 11 participants had higher power output in H-BR vs. H-PLA, while only 6 out of 11 had higher power output in N-BR vs. N-PLA (Figure 3). In support of a small effect of BR, a recent

meta-analysis, including studies performed in hypoxia and normoxia, reported a non-significant 0.8% improvement in time trial endurance performance following BR supplementation [55]. The improvement in 10-km TT completion time and power output of 0.6% and 1.6%, respectively, in the present study, is of practical relevance for elite and well-trained athletes. Specifically, only 0.9% separated first and fourth position during the 13.8-km TT of stage 1 at the 2015 Tour De France cycling race [56], and only 0.3% separated the first and third position during the 9.7-km TT of stage 1 at the 2018 Giro d'Italia cycling race [57]. Further, 0.6% is the smallest worthwhile change in completion time for road TT cyclists proposed by Paton and Hopkins [58].

Few other studies have examined the effects of  $\text{NO}_3^-$  on TT performance in well-trained athletes in both normoxia and hypoxia within the same study. None of these studies have reported significant improvements in TT performance after BR supplementation [36,38,39]. Nonetheless, the study by Bourdillion et al. [39] reported statistically non-significant improvements in 15-km TT performance of 16s (~1%) and 151s (~7%) in normoxia and hypoxia, respectively.

In general, studies on TT performance performed in well-trained athletes in hypoxia or in normoxia have reported mixed results. In hypoxia, two studies found statistically significant improvements of 2.2-3.2% (~2.2%) [17,21], while one study reported no effect [37]. In normoxia, numerous studies show no effect [22-27,59-61], while a few studies report a significant effect [15,16,20]. The discrepancy in the literature may partly be due to the use of different  $\text{NO}_3^-$  supplementation strategies that vary in terms of source, dose, and duration (e.g., chronic vs. acute). Many of the previous TT studies have not used an optimized

supplementation strategy. Specifically, some studies have used sodium nitrate as the source of  $\text{NO}_3^-$  [23,39], while there is evidence suggesting that supplementation with  $\text{NO}_3^-$  in concentrated BR is more effective [40,62]. Several studies have used an acute dose of BR [17,25,26,36-38,59-61], however, a chronic loading protocol consisting of BR supplementation over several days, as used in the present study, has been suggested to be more effective in raising plasma levels of  $\text{NO}_3^-$  and  $\text{NO}_2^-$ , and improving performance [11,43]. Finally, several studies have used a low-to-moderate dose of  $\text{NO}_3^-$  [36,37,59-61], while a higher dose (8-16 mmol), as used in the present study, may be more effective in raising plasma levels and improving performance [13,20,44]. The high dose of  $\text{NO}_3^-$  used in the present study was tolerated without any adverse events or complaints, demonstrating the efficacy of this supplementation strategy for 7 days. However, there is currently no evidence demonstrating additional benefits with doses higher than 8 mmol. In support of the notion that supplementation strategy is important, studies utilizing an optimized supplementation strategy with chronic supplementation of high dose  $\text{NO}_3^-$  in the source of BR have reported a significant 2.1% [16] and a non-significant 1.7% [24] improvement in TT power output in trained cyclists.

#### 4.1.2 Plasma levels of $\text{NO}_3^-$ and $\text{NO}_2^-$

In the present study, plasma levels of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  after placebo (i.e., nitrate-depleted BR) supplementation, were similar to results from other studies using nitrate-depleted BR [17,21,22,37,38,63].

Four and seven days of BR supplementation increased  $\text{NO}_3^-$  and  $\text{NO}_2^-$  to levels reported in studies using a similar supplementation strategy [13,22], with no

377 differences between 4 and 7 days. Notably,  $\text{NO}_3^-$  and  $\text{NO}_2^-$  levels, in the present  
 378 study, were higher than those reported in studies using acute supplementation  
 379 [17,21,37,38,63] ) or lower dosage of  $\text{NO}_3^-$  [17,37,59,60]. Taken together,  
 380 markedly elevated levels of  $\text{NO}_3^-$  and  $\text{NO}_2^-$ , in the present study, indicate that BR  
 381 supplementation was effective in providing an abundant source of NO via the  
 382 nitrate-nitrite-NO pathway. Plasma levels of nitrite displayed a higher variability  
 383 compared to plasma nitrate (Fig 2 and Fig 3). This is a common finding and is  
 384 most likely due to the shorter half-life of nitrite (less than 1h)[64] compared to  
 385 nitrate (5-8h)[65]. This may be explained by a much higher reactivity of nitrite  
 386 being subjected to both enzymatic reduction to NO and oxidation to nitrate [33].  
 387 Moreover, due to the markedly lower concentration of nitrite in plasma,  
 388 measuring techniques display more variable results compared to nitrate.

#### 389 *4.1.3 Physiological effects of beetroot juice supplementation*

390 We found a main effect of supplementation on  $\text{VO}_2$ , VE,  $\text{VCO}_2$  and %  $\text{VO}_{2\text{max}}$   
 391 such that BR supplementation resulted in higher  $\text{VO}_2$ , VE,  $\text{VCO}_2$  and %  $\text{VO}_{2\text{max}}$   
 392 during the TT in both hypoxia and normoxia. As studies generally show  
 393 unchanged [10,12,13,30] or reduced [66,67]  $\text{VO}_{2\text{max}}$  following BR  
 394 supplementation, these results indicate that the participants were able to utilize a  
 395 higher proportion of their maximal aerobic capacity during the TT with BR.  
 396 Further, in the present study, a proxy of exercise efficiency ( $\text{PO}/\text{VO}_2$ ) during the  
 397 TT was unaffected by BR supplementation, suggesting that changes in exercise  
 398 efficiency did not contribute to improved TT performance. In agreement with this,  
 399 several studies, in well-trained athletes ( $>60 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ ), have shown  
 400 unchanged exercise efficiency during submaximal exercise following BR

401 supplementation [24,37,38,63], while only a single study has reported improved  
 402 efficiency (lower  $\text{VO}_2$  during submaximal exercise) in well-trained athletes [21].  
 403 In club-level cyclists ( $56.0 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ ) [15], BR supplementation improved  
 404 power output with unchanged  $\text{VO}_2$  (greater  $\text{PO}/\text{VO}_2$ ), indicating improved  
 405 exercise efficiency. The discrepancy between these results could be due to the  
 406 training level of the subjects, as our study included well-trained athletes ( $66.4$   
 407  $\text{ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ ). Thus, the increase in  $\% \text{VO}_{2\text{max}}$  with BR was likely the main factor  
 408 contributing to increased TT performance. In accordance with these results,  
 409 Bourdillion et al. [39] reported greater  $\text{VO}_2$  and VE with nitrate supplementation  
 410 in trained cyclists during a 15-km TT in normoxia and hypoxia, which was  
 411 accompanied by a non-significant increase (1-7%) in performance (discussed  
 412 above). Contributing to the increased  $\text{VO}_2$  with BR, the increase in VE ( $\sim 6\text{L}/\text{min}$ )  
 413 is estimated to account for 10-15  $\text{ml}/\text{O}_2/\text{min}$  ( $\sim 10\text{-}20\%$ ) of the increase in  $\text{VO}_2$ ,  
 414 due to greater oxygen demands of the respiratory muscles [68-70].  
 415 The active skeletal muscles are the primary site for  $\text{O}_2$  usage during the TT, and  
 416 oxygenation in the vastus lateralis was monitored continuously using NIRS.  
 417 During the TT,  $\Delta\text{HHb}$  increased in hypoxia compared with normoxia, indicating  
 418 increased  $\text{O}_2$  extraction. However, in agreement with Kelly et al. [30] and  
 419 Bourdillion et al. [39],  $\Delta\text{HHb}$  was unaffected by BR supplementation, indicating  
 420 that fractional  $\text{O}_2$  extraction in vastus lateralis was not different between BR and  
 421 PLA. Hence, according to the Fick principle, the increased oxygen uptake in the  
 422 present study may be a result of increased total  $\text{O}_2$  extraction due to increased  
 423 blood flow. This interpretation is consistent with results demonstrating that  $\text{NO}_3^-$

424 supplementation enhances vascular control and muscle blood flow redistribution  
425 during exercise [8,49,72].

## 426 **5.1 Conclusion**

427 In summary, our results provide novel evidence that chronic high-dose BR  
428 supplementation improves 10 km time trial performance of well-trained cyclists in  
429 both normoxia and hypoxia. Further, BR supplementation resulted in higher  $\text{VO}_2$   
430 and VE during the TT, suggesting that utilization of a greater proportion of the  
431 aerobic capacity contributed to the improved performance. While our results do  
432 not identify the underlying mechanisms, enhanced vascular control and muscle  
433 blood flow redistribution may contribute to higher  $\text{VO}_2$  and improved time trial  
434 performance with BR supplementation.

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## 441 **7.1 Conflict of interest statement**

442 The authors declare: no support from any organization for the submitted work; no  
443 financial relationships with any organizations that might have an interest in the  
444 submitted work in the previous 3 years; no other relationships or activities that  
445 could appear to have influenced the submitted work. EW is a co-applicant on  
446 patents related to the therapeutic use of nitrate and nitrite.

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- High-dose  $\text{NO}_3^-$  supplementation improved time trial performance of cyclists
- Oxygen uptake during the time trial was elevated with  $\text{NO}_3^-$  supplementation
- The effects of  $\text{NO}_3^-$  supplementation were not different between hypoxia and normoxia